(12) UK Patent Application (19) GB (11) 2 145 992 A

(43) Application published 11 Apr 1985

(21) Application No 8422052

(22) Date of filing 31 Aug 1984

(30) Priority data

(31) 528395 (32) 1 Sep 1983 (33) US

(51) INT CL[4]
B01J 13/02

(52) Domestic classification
B8C A

(56) Documents cited
GB A 2046209

(58) Field of search
B8C

(71) Applicant
Damon Biotech Inc (USA-Delaware),
119 Fourth Avenue, Needham Heights,
Massachusetts 02194, United States of America

(72) Inventor
Allan P. Jarvis

(74) Agent and/or Address for Service
Graham Watt & Co.,
Riverhead, Sevenoaks, Kent TN13 2BN

(54) Microencapsulation of viable cells

(57) A process for damage-free encapsulation of a variety of core materials including viable cells involves suspending the core material in an aqueous solution of a polysaccharide containing cationic groups, such as an aminated glucopolysaccharide. A temporary matrix is formed by gelling droplets of the suspension using a solution containing a divalent or multivalent anion. The temporary matrix is permanently cross-linked with a polymeric material containing plural anionic groups, e.g., polyaspartic or polyglutamic acid, to form a semipermeable membrane. The interior of the microcapsule may be resolubilized by subjecting it to a solution of low molecular weight cations.

GB 2 145 992 A

SPECIFICATION

Microencapsulation of core materials e.g. viable cells

The present invention relates to microencapsulation of core materials, e.g. viable cells.

More particularly, this invention relates to the encapsulation of core material within a semipermeable membrane and provides an improved process for encapsulating, e.g. pH, temperature, or ionic strength-sensitive core material, including viable cells, within a microcapsule. The encapsulation process disclosed herein enables formation of a semipermeable membrane without damaging the core material. The invention also relates to a capsule having an aminated polymeric inner layer ionically bound to an anionic polymeric outer layer.

Although a number of processes for microencapsulation of core material have been developed, most of these processes cannot be used for pH, temperature or ionic strength-sensitive material such as viable cells because of the harsh conditions necessary for encapsulation. U.S. Patent No. 4,352,883 discloses what is believed to be the first process for successfully encapsulating viable tissue or cells within a semipermeable membrane. In the patented process, a temporary capsule of a gellable material, preferably an anionic gum such as sodium alginate, is formed about the tissue or cells and a permanent, semipermeable membrane is formed by cross-linking surface layers of the temporary capsule. In the process, a mixture of the gum and the core material is subjected to a gelling solution, preferably a calcium ion solution, to produce a temporary capsule. The resulting temporary capsule is reacted with a solution of a polycationic material to form a permanent membrane. The interior of the capsule may be reliquified by reestablishing conditions under which the anionic gum is liquid, e.g., changing the ionic environment by placing the capsules in phosphate buffered saline. Reliquification of the interior of the capsule facilitates nutrient transport across the membrane, promoting cell growth. The process need not damage the core material or hamper the viability of cells because the temperature, ionic strength, and pH ranges used in the encapsulation process need not be harsh.

An object of the present invention is to provide an improved process for encapsulating viable cells or other fragile material in semipermeable membranes, e.g. for encapsulating core materials which are difficult to capsulate using known procedures because of pH, ionic strength, charge, or temperature sensitivity. A further object of the invention is to provide an improved capsule comprising a semipermeable membrane.

According to one aspect, the present invention provides a process for encapsulating a core material within a semipermeable membrane, comprising the steps of: A. suspending the core material in an aqueous medium in which is dissolved a polysaccharide comprising cationic groups, B. forming the suspension into a droplet containing the core material, C. subjecting the droplet to a solution of anions to gel the droplet as a discrete, shape-retaining temporary matrix, and D. cross-linking surface layers of the temporary matrix to produce a capsule about said droplet, by subjecting the temporary matrix to a polymer containing anionic groups reactive with the said cationic groups.

Thus, the invention provides, inter alia a process for encapsulating a viable cell within a semipermeable membrane, comprising the steps of: A. suspending the cell in an aqueous medium compatible with the viability of said cell, and the said medium containing an aminated glucopolysaccharide; B. forming the suspension into a droplet containing said cell; C. subjecting the droplet to a gelling solution comprising an aqueous solution of anions to gel the droplet and to form a shape-retaining, water-insoluble temporary matrix, and D. permanently cross-linking a surface layer of said temporary matrix to produce a membrane about said droplet by subjecting the temporary matrix to a polymer containing a plurality of carboxyl groups.

According to a second aspect, the present invention provides a capsule comprising a membrane defining an enclosed intracapsular volume, the membrane consisting essentially of an inner layer comprising a polyaminated polymer and an outer layer comprising a polyanionic polymer, the said polyaminated and polyanionic polymers being crosslinked by ionic interaction between cationic amine groups on the polyaminated polymer and anionic groups on the polyanionic polymer to form a water-insoluble permeable capsule.

In one aspect, the invention features a process for encapsulating core material such as enzymes, antibodies, hormones, or viable cells, e.g. tissue culture or genetically modified cells, within a semipermeable membrane. The core material is suspended in an aqueous medium having dissolved therein a polysaccharide containing cationic groups, preferably an aminated polysaccharide such as poly D Glucosamine (chitosan). A droplet of the suspension is gelled by subjecting the droplet to a solution of a divalent or multivalent anions, e.g., $PO_4^=$, $HPO_4^=$, or $SO_4^=$ salts. A permanent membrane is formed by cross-linking surface layers of the temporary matrix with a polymer containing anionic, preferably carboxyl groups reactive with the cationic groups of the matrix. Poly-L-glutamic acid and poly-L-aspartic acid, either as acids or salts, are preferred anionic polymers. The interior of the capsule may be resolubilized by subjecting the capsule to a solution of low molecular weight polycations, e.g., spermadine, spermine, or urea. Reliquification of the interior promotes mass transport between extracapsular fluid and the intracapsular volume.

In another aspect, the invention features a process for encapsulating a viable cell within a

semipermeable membrane. The cell is suspended in an aqueous medium compatible with its viability containing an aminated glucopolysaccharide. A droplet of the suspension is subjected to a gelling solution thereby forming a shape-retaining, water-insoluble temporary matrix. The gelling solution is an aqueous solution of a di- or multivalent anions, e.g., phosphate, monohydric phosphate, or sulfate salts. Surface layers of the temporary matrix are permanently cross- linked by subjecting the capsule to a polymer containing a plurality of carboxyl groups, e.g., polyglutamic acid or polyaspartic acid. The interior of the capsule is reliquified in a substantially precipitant-free reaction by subjecting the capsule to a solution of a low molecular weight polycation, e.g., spermadine, spermine, or urea. Reliquification occurs by removal of multivalent anions from the interior gel. Mammalian tissue cells in a tissue growth medium and genetically modified prokaryotic or eukaryotic cells in a growth medium may be encapsulated by this process. The encapsulation procedure is gentle so the encapsulated cell is not damaged and can undergo normal metabolic processes including growth and reproduction within the capsule.

In a further aspect, the invention features a capsule having a membrane defining an enclosed intracapsular volume. The membrane has an inner layer of a polyaminated polymer, preferably an aminated polysaccharide, most preferably an aminated glucopolysaccharide, ionically cross-linked to an outer layer of a polyanionic polymer, preferably a polycarboxylated polymer such as polyglutamic acid or polyaspartic acid, to form a water-insoluble permanent capsule. A cell, preferably a eukaryotic, bacterial, or genetically modified cell, may be disposed within the intracellular volume. If a cell is disposed within the intracellular volume, the polyanionic and polyaminated polymers should be physiologically compatible with the cell.

The invention will now be explained in more detail in the following exemplary description by reference to preferred embodiments thereof.

The present invention provides a process for encapsulating core materials such as viable eukaryotic or prokaryotic, naturally occurring or genetically modified, cells or tissue culture within a semipermeable membrane. The invention also provides a new type of multi-layer capsule.

A gel or temporary matrix is formed about the core material or viable cell by reacting a polysaccharide or glucopolysaccharide containing cationic groups with a multivalent anion. Surface layers of the resulting temporary matrix are then ionically cross-linked by a polymer containing anionic, preferably carboxyl, groups forming a permanent membrane encapsulating the core material. The interior of the capsule may be reliquified by subjecting the capsule to a solution of low molecular weight polycations.

Core material, for example, enzymes, hormones, antibodies or viable cells, is suspended in an aqueous medium containing a gellable, cationic group-containing polymer. The preferred gellable polymer is an aminated glucopolysaccharide, most preferably chitosan (poly-D-glucosamine). Chitosan is formed by acid hydrolysis of chitin (poly-D-N-acetylglucosamine), the primary building material of invertebrate exoskeletons. Chitosan is a long chain, aminated polymer which is physiologically compatible with most cells. It is only slightly soluble in water but can be readily dissolved in dilute acetic acid.

Stock solutions of chitosan are prepared by dissolving the chitosan in 0.5 molar acetic acid. The acetic acid is removed from the solution by repeated dialysis against phosphate buffered saline (PBS). Chitosan does not precipitate out of this solution if the solution is kept below 37°C. The preferred stock solution, 1.4- chitosan in PBS, has been stored successfully at 4°C for extended periods.

Chitosan prepared as described above is mixed with the material to be encapsulated, forming a solution or slurry, before it is formed into droplets or other shapes. Although solutions having a final chitosan concentration of 0.4% (w/v) will gel, optimum gelling is obtained with 0.8-1.2% (w/v) chitosan in isotonic, 125 mM $Na_2 HPO_4$. Droplets of the chitosan/core material suspension can be formed by any conventional droplet-forming apparatus. One such droplet- forming apparatus is described below.

A tube containing the suspension is fitted with a stopper which holds a droplet-forming apparatus. The apparatus consists of a housing having an upper air intake nozzle and an elongate hollow body friction fitted into the stopper. A 10cc syringe equipped with a stepping pump is mounted atop the housing with a needle, e.g., a 0.01 inch (0.25 mm) I.D. Teflon coated needle, passing through the length of the housing. The interior of the housing is designed such that the tip of the needle is subjected to a constant laminar air flow which acts as an air knife. In use, the stepping pump is actuated to incrementally force droplets of solution from the tip of the needle. Each drop is "cut off" by the air stream and falls approximately 2.5cm into the gelling solution, preferably a disodium hydrogen phosphate solution, where it is immediately gelled by reaction with the negative ions. The distance between the tip of the needle and the surface of the gelling solution is preferably great enough to allow the chitosan/core material suspension to assume the most physically favorable shape: a sphere (maximum volume for a minimum surface area). Air from the tube bleeds through an opening in the stopper. This procedure results in a "cross-linking" of the gel and the formation of a high viscosity shape-retaining protective temporary matrix containing the suspended core material and its medium. The temporary matrices collect in the solution as a separate phase and may be separated by aspiration.

The preferred multivalent gelling solution is a 125mM Na_2HPO_4 solution; however, monobasic sodium phosphate and sodium sulfate solutions have also produced acceptable temporary matrices. Sodium citrate can gel the suspension but best results are obtained with monobasic or dibasic phosphate and sulfate solutions. If the anion level is too low (e.g., below approximately 100 mM), gelling may not occur.

Temporary matrices formed by this process are collected and washed to remove excess gelling solution. The matrices are then subjected to a coating or cross-linking solution of a polyanionic, preferably polycarboxylated polymer. A preferred cross-linking solution is a 1% solution of poly-L-aspartic acid of poly-L-glutamic acid, diluted 1:15 with 150 mM sodium chloride, yielding a final polymer concentration of 6.6×10^{-4} g/100 ml. The molecular weight of the poly-L-aspartic or poly-L-glutamic acid can range from 3,000-100,000 daltons or higher, but the 25,000-60,000 dalton range is preferred. A reaction time of 3-6 minutes at ambient room temperature has been found to be satisfactory.

The following non-limiting examples set forth exemplary procedures for practice of the invention.

Example 1

A stock solution of 1.4% chitosan (CSN-Sigma Chemical Co.) in 14-17 mM (w/v) isotonic PBS, prepared as previously described, was used in this experiment. Different concentrations of CSN were tested to determine the optimum CSN concentration for temporary matrix formation. In each case, 1 ml samples were formed from the CSN stock solution and fetal calf serum (FCS-flow Laboratories). Droplets were produced and introduced into solutions as described below using a droplet-forming apparatus as previously described. Table 1 illustrates three different CSN concentrations (w/v) tested in this experiment, 0.6%, 0.8% and 1%.

TABLE 1

Sample No.	*CSN-ml*	*FCS ml*	*Concentration CSN (w/v)*
1	0.57	0.43	0.8%
2	0.42	0.58	0.6%
3	0.72	0.28	1.0%

Two gelling buffers were prepared, 110mM sodium citrate (Sigma), and 125mM disodium hydrogenphosphate (Sigma). In all cases, the temporary matrices formed using these buffered solutions were acceptable, but the sodium citrate buffer produced inadequate gelling of sample No. 1. While all of the samples formed acceptable temporary matrices in the disodium hydrogen phosphate buffer, sample 3 provided the best gelling.

A 1% solution of poly-L-aspartic acid (40,000 dalton molecular weight-Sigma), was diluted 1:15 with 150mM sodium chloride (Sigma) to form a 6.6×1010^{-4} g/100 ml solution. Temporary matrices formed from sample 3 were removed from the gelling buffer, washed repeatedly with isotonic PBS, and resuspended in the poly-L-aspartic acid solution. After six minutes at room temperature in the poly-L-aspartic acid solution, the cross-linking reaction was complete, forming the permanent membranes. The capsules were substantially spherical, about 380-480 microns in diameter. Some capsules had small tails. Capsules formed by this process were not sticky and had no tendency towards clumping. Porosity of the capsules was determined empirically to be about 80,000 daltons. This example illustrates that acceptable capsules can be formed using the process of this invention.

Example 2

This example was conducted to demonstrate that the viability of cells is not impaired by the encapsulation process. The cell culture used in this experiment was the Friend Erythroleukemic cell line (FEL_{745}), a mouse erythroleukemic line which grows readily in a suspension culture.

A culture of FEL_{745} cells was centrifuged for 5 minutes and the resulting pellet was resuspended as a slurry in fetal calf serum (Flow Labs). A stock solution of 1.4% chitosan was prepared as previously described and mixed with the cell culture solution, yielding a final chitosan concentration of 1% (w/v) and a cell concentration of about 5×10^6 cells/ml. Temporary matrices were produced by forcing the chitosan-cell mixture through a droplet-forming apparatus (previously described) and contacting the resulting liquid microspheres with the 125mM (w/v) phosphate ion solution previously described. The resulting temporary matrices were washed repeatedly with isotonic PBS and resuspended in 6.6×10^{-4} g/100 ml (w/v) solution of poly-L-aspartic acid (40,000 daltons molecular weight) in 150mM sodium chloride. After six minutes at room temperature in the poly-L-aspartic acid solution, the resulting capsules were washed twice with the culture medium RPMI-1640 (Flow Labs) containing 10% fetal calf serum and antibiotics. The microcapsules were placed into tissue culture flasks with the culture medium and incubated at 37°C at a 5% CO_2 atmosphere.

Samples of the cell culture were removed at various intervals. Examination under a microscope showed that the cells were growing and reproducing, illustrating cell viability after the encapsulation procedure. As may be noted, there was no reliquification step used since Friend cells can grow in a gel culture as well as a suspension culture. However, many types of cells need a fluid culture to reproduce. Cell viability should not be affected by the resolubilization of the capsule interiors. The capsule provides a microenvironment free from external contamination.

CLAIMS

1. A process for encapsulating a core material within a semipermeable membrane, comprising the steps of: A. suspending the core material in an aqueous medium in which is dissolved a polysaccharide comprising cationic groups, B. forming the suspension into a droplet containing the core material, C. subjecting the droplet to a solution of anions to gel the droplet as a discrete, shape-retaining temporary matrix, and D. cross-linking surface layers of the temporary matrix to produce a capsule about said droplet, by subjecting the temporary matrix to a polymer containing anionic groups reactive with the said cationic groups.

2. The process according to claim 1, wherein the polysaccharide containing cationic groups is an aminated polysaccharide.

3. The process according to claim 2, wherein the aminated polysaccharide is chitosan.

4. The process according to claim 1, 2 or 3, wherein the anions in said solution thereof are selected from phosphate, dibasic phosphate, sulfate, and mixtures thereof.

5. The process according to claim 4 wherein the said solution of anions is prepared by dissolving the salt containing the said anion in an aqueous solution.

6. The process according to any preceding claim, wherein the said polymer contains carboxyl groups as the said anionic groups.

7. The process according to claim 6, wherein the said polymer is selected from polyaspartic acid, polyglutamic acid, salts thereof and mixtures thereof.

8. The process according to any of claims 1 to 7, further comprising an additional step of resolubilizing the gel within the capsule.

9. The process according to claim 8, wherein the gel is resolubilized by subjecting the capsule to a solution of a low molecular weight polycation.

10. The process according to claim 9, wherein the low molecular weight polycation is selected from the cations of spermadine, spermine, urea, and mixtures thereof.

11. The process according to any of claims 1 to 10, wherein the core material is selected from enzymes, antibodies, hormones, and viable cells.

12. The process according to claim 11, wherein the viable cells comprise a tissue culture or individual cells thereof and the aqueous medium in which they are suspended comprises a tissue culture medium.

13. The process according to claim 11 or claim 12, wherein the viable cells comprise genetically modified cells.

14. A process for encapsulating a viable cell within a semipermeable membrane, comprising the steps of: A. suspending the cell in an aqueous medium compatible with the viability of said cell, and the said medium containing an aminated glucopolysaccharide; B. forming the suspension into a droplet containing said cell; C. subjecting the droplet to a gelling solution comprising an aqueous solution of anions to gel the droplet and to form a shape-retaining, water-insoluble temporary matrix, and D. permanently cross-linking a surface layer of said temporary matrix to produce a membrane about said droplet by subjecting the temporary matrix to a polymer containing a plurality of carboxyl groups.

15. The process according to claim 14, comprising an additional step of reliquifying the interior of the membrane by subjecting the product of step D to a solution of a low molecular weight polycation which removes anions from the gel by a substantially precipitant-free reaction.

16. The process according to claim 14, wherein the gelling solution is an aqueous salt solution the anion of which is phosphate, dibasic phosphate, sulfate, and mixtures thereof.

17. The process according to claim 14, 15 or 16, wherein the polymer containing a plurality of carboxyl groups is selected from polyaspartic acid, polyglutamic acid, salts thereof, and mixtures thereof.

18. The process according to claim 15 or any claim dependent on claim 15, wherein the low molecular weight polycation is selected from the cations of spermadine, spermine, and mixtures thereof.

19. The process according to claim 18, wherein the viable cell comprises a mammalian tissue cell and the said aqueous medium comprises a tissue growth culture.

20. The process according to claim 18, wherein the viable cell comprises a genetically modified cell and the said aqueous medium comprises a growth medium.

21. A process according to claim 1 and substantially as hereinbefore described.

22. A process according to claim 14 and substantially as hereinbefore described.

23. Encapsulated material prepared by the process claimed in any of claims 1 to 22.

24. A capsule comprising a membrane defining an enclosed intracapsular volume, the membrane consisting essentially of an inner layer comprising a polyaminated polymer and an outer layer comprising a polyanionic polymer, the said polyaminated and polyanionic polymers being crosslinked by ionic interaction between cationic amine groups on the polyaminated polymer and anionic groups on the polyanionic polymer to form a water-insoluble permeable capsule.

25. The capsule according to claim 24, wherein the polyaminated polymer is an aminated polysaccharide.

26. The capsule according to claim 25, wherein the aminated polysaccharide is a polyaminated glucopolysaccharide.

27. The capsule according to claim 24, 25 or 26, wherein the polyanionic polymer comprises a polycarboxylated polymer.

28. The capsule according to claim 27, wherein the polyanionic polymer is polyaspartic acid or a salt thereof.

29. The capsule according to claim 27, wherein the polyanionic polymer is polyglutamic acid or a salt thereof.

30. The capsule according to any of claims 24 to 29, which contains a cell disposed in a culture medium for the cell within the intracapsular volume.

31. The capsule according to claim 30, wherein the said polyaminated and polyanionic polymers are physiologically compatible with the cell.

32. The capsule according to claim 30, wherein the cell comprises a eukaryotic cell.

33. The capsule according to claim 30, wherein the cell comprises a genetically modified cell.

34. A capsule according to claim 24 and substantially as hereinbefore described.

Printed in the UK for HMSO, D8818935, 2/85, 7102.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.